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Properties of a *meq*-Deleted rMd5 Marek's Disease Vaccine: Protection Against Virulent MDV Challenge and Induction of Lymphoid Organ Atrophy Are Simultaneously Attenuated by Serial Passage *In Vitro*

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SUMMARY. We have previously shown that deletion of the *meq* gene from the genome of Cosmid-cloned rMd5 strain of Marek's disease virus (MDV-1) resulted in loss of transformation and oncogenic capacity of the virus. The rMd5ΔMeq (Meq null) virus has been shown to be an excellent vaccine in maternal antibody positive (MAb+) chickens challenged with a very virulent plus (vv+) strain of MDV, 648A. The only drawback was that it retained its ability to induce bursa and thymus atrophy (BTA) like that of the parental rMd5 in maternal antibody negative (MAb−) chickens. We recently reported that the attenuated Meq null virus did not induce BTA at the 40th cell culture passage onward. Its protective ability against challenge with vv+ MDV, strain 686 was similar to the original virus at the 19th passage in MAb− chickens. In this study, we compared the same series of attenuated *meq* null viruses in commercial chickens. In commercial chickens with MAb, the attenuated viruses quickly lost protection with increasing cell culture attenuation. These data suggest that although attenuation of these *meq* null viruses eliminated BTA, it had no influence on their protective efficacy in MAb− chickens. However, in commercial chickens (MAb+), the best protection was provided by the original 19th passage; the attenuated 40th passage was as good as one of the currently commercial CVI988/Rispens vaccine, and it did not induce BTA. Therefore, protection against virulent MDV challenge and induction of lymphoid organ atrophy are simultaneously attenuated by serial passage *in vitro*.

RESUMEN. Propiedades de la vacuna rMd5 con deleción del gene *meq*: contra la enfermedad de Marek: La protección contra el desafío virulento y la inducción de la atrofia de órganos linfoides son simultáneamente atenuadas por pasajes seriados *in vitro*.

Se ha demostrado previamente que la supresión del gene *meq* del genoma de la cepa rMd5 del virus de la enfermedad de Marek serotipo 1 (MDV-1) clonada en cósmidos resultó en la pérdida de la capacidad de transformación y oncogénica del virus. El virus rMd5ΔMeq (con el gene Meq nulificado) ha demostrado ser una excelente vacuna en pollos con anticuerpos maternos (MAb+) y desafiados con una cepa muy virulenta plus (vv+), la cepa 648A. El único inconveniente era que conservaba su capacidad de inducir atrofia del timo y de la bolsa de Fabricio como la observada con la cepa rMd5 original en pollos sin anticuerpos maternos (MAB-). Recientemente, se reportó que el virus atenuado con el producto Meq nulo, no indujo atrofia del timo ni de la bolsa a partir del pasaje 40 en cultivos celulares. Su capacidad de protección contra el desafío con un virus de Marek muy virulento plus, cepa 686 era similar al virus original en el paso 19a en pollos sin anticuerpos maternales. En este estudio, se comparó la misma serie de virus atenuados con el gene *meq* nulificado en pollos comerciales. En los pollos comerciales con anticuerpos maternales, los virus atenuados perdieron rápidamente su capacidad de protección con el pasaje de atenuación consecutivo en cultivo celular. Estos datos sugieren que aunque la atenuación de estos virus con el gene *meq* nulificado eliminó la capacidad de inducir atrofia del timo y de la bolsa, no tenía ninguna influencia sobre su eficacia protectora en pollos sin anticuerpos maternos. Sin embargo, en pollos comerciales (con anticuerpos maternos), la mejor protección fue proporcionada por el pasaje original 19; el pasaje atenuado 40 fue tan bueno como la vacuna comercial actual CVI988/Rispens y no indujo atrofia del timo ni de la bolsa de Fabricio. Por lo tanto, la protección contra el desafío virulento de la enfermedad de Marek y la inducción de la atrofia de los órganos linfoides son simultáneamente atenuadas mediante pasajes seriados *in vitro*.

Key words: Marek's disease virus, Meq null virus, rMd5ΔMeq, vaccine, chicken

Abbreviations: ADOL = Avian Disease and Oncology Laboratory; BAC = bacterial artificial chromosome; BTA = bursa thymus atrophy; DEF = duck embryo fibroblast; HVT = turkey herpesvirus; MAb = maternal antibody; MD = Marek's disease; MDV = Marek's disease virus; Meq = MDV EcoQ; PFU = plaque-forming units; rMd5 = recombinant Md5 virus; rMd5ΔMeq = Meq null rMd5 virus; p = passage; PI = protective index; vv = very virulent; vv+ = very virulent plus

Marek's disease virus serotype 1 (MDV-1) is a member of gallid herpesvirus 1. The *Eco*R1-Q fragment of the viral genome encodes a *meq* (MDV EcoQ) gene and was first identified in MDV tumor and tumor cell lines in 1988 (30). A survey of transcription from MDV-1 in various tumor cell lines revealed the presence of large amounts of a family of RNAs complementary to the *Eco*RI fragment. This fragment was not highly transcribed in infected-duck embryo fibroblasts (DEFs). The portion of the *Eco*RI-Q fragment that hybridized with the RNAs was sequenced. Analysis of the amino acid translation of this region of DNA suggested that these transcripts may code for a protein homologous to *jun/fos* oncogenes (5,30). *meq* is a unique gene, present only in serotype 1 MDV and is consistently expressed in latently infected or tumor cells. The biochemical structure and functional studies of MEQ protein were well documented by several groups of researchers (1,5,8,12,13,17). *meq*

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encodes a protein that is 339 amino acids long and contains domains for DNA binding, dimerization, and transactivation/repression activities (5,17). The basic DNA binding and the leucine zipper regions at the N-terminal region of *meq* are closely related to the *jun/fos* oncoproteins, while the C-terminal proline rich domain structurally resembles the WT-1 tumor suppressor gene (8). The *meq* gene has been deleted from the very virulent rMd5 genome, and the nononcogenic mutant virus was named rMd5ΔMeq (14). We have conducted vaccine efficacy experiments in Avian Disease and Oncology Laboratory (ADOL) $15I_5 \times 7_1$ maternal antibody positive (MAb+) chickens and showed that the rMd5ΔMeq virus protected better than CVI988/Rispens following challenge with 648A, a highly pathogenic (very virulent plus [vv+]) strain of MDV-1 (11). Three large-scale field experiments, in which seeder birds were challenged with the most virulent vv+ 686, also showed that rMd5ΔMeq was a superior vaccine under field conditions (10). All these data indicated that the *meq* null virus provided protection better than all of the commercial CVI988/Rispens vaccines following challenge with vv+ MDV. Recently, a bacterial artificial chromosome (BAC)-cloned rMd5ΔMeq virus, also shown to be nonpathogenic, provided protection better than CVI988/Rispens when challenged with a vv+ strain of MDV (25).

We have compared the 19th passage (p) of rMd5ΔMeq/p19 vaccine in both MAb+ and maternal antibody negative (MAb−) chickens and found significant lymphoid organ (bursa and thymus) atrophy (BTA) and body weight loss in MAb− chickens but not in MAb+ chickens (9). The parental rMd5 virus is a very virulent (vv) pathotype of MDV-1 (18) capable of inducing BTA even in MAb+ chickens (9). The rMd5ΔMeq/p19 virus was shown to be an effective vaccine candidate in MAb− chickens, but with the drawback of induction of BTA and body weight loss in MAb− chickens like that of parental rMd5 virus. The rMd5ΔMeq/p19 virus, however, did not induce BTA in MAb+ commercial chickens and therefore could be a candidate vaccine.

The fact that rMd5ΔMeq/p19 virus showed lymphoid organ atrophy in MAb− chickens is considered a safety issue and will undoubtedly interfere with commercialization and licensing of this candidate vaccine. In order to improve the safety of rMd5ΔMeq/p19 vaccine, we hypothesized that BTA will be eliminated by attenuation of this virus in cell culture. We generated 50 cell culture passages of attenuated rMd5ΔMeq viruses and found no significant lymphoid organ atrophy beginning at p40 onward when compared with control un-inoculated chickens. The protective ability of these attenuated *meq* null viruses against challenge with vv+ 686 MDV is similar to the virus at p19 in MAb− chickens. Thus cell-culture attenuation of *meq* null rMd5 eliminated the capacity of the virus to induce BTA without any apparent negative influence on its protective efficacy. This attenuation rendered the virus safe to use even in MAb− chickens, a characteristic that should facilitate commercialization and licensing by vaccine manufacturers. In this paper, we report our studies on the protective efficacy of attenuated rMd5ΔMeq virus in commercial chickens.

MATERIALS AND METHODS

Chickens. The trial in commercial chickens was conducted in White Leghorn chickens of the Hy-Line International® VarietyW-36, West Des Moines, IA. The W-36 chicks were from MD-vaccinated parents and were considered to be MAb+. MAb− chickens used were from MD-susceptible F1 progeny of ADOL line 151_5 males and line 7_1 dams derived from a breeding flock maintained under specific-pathogen-free conditions, and lack antibodies to many avian pathogens including MDV, turkey herpesvirus (HVT), avian leukosis virus, and reticuloendotheliosis virus as determined by periodic serological tests.

Cells and viruses. Primary DEF were used for virus propagation. The HVT (38) and SB-1 (21) viruses were propagated in chicken embryo fibroblasts. Recombinant rMd5ΔMeq virus (14), which lacks both copies of the *meq* oncogene, and the parental recombinant rMd5 virus were generated from cosmids derived from the very virulent Md5 strain as previously described (18). The rMd5ΔMeq construct at p19 was serially passaged in DEF cell cultures. The rMd5ΔMeq preparations used in this trial as follows: p19, p36, p40, and p50. The process of attenuation of these viruses is described in the following section. The standard vaccine virus CVI988/Rispens strain (19,20) of serotype 1 was from ADOL obtained originally from Merial Select. The vv+ 686 (p10) strain of serotype 1 MDV (32) was used as challenge virus. Additional vaccines used in the commercial trial were as follows: bivalent HVT+SB1, CVI988/Rispens (A), and CVI988/Rispens (B). These three vaccines were commercial vaccines from different manufacturers.

Attenuation of rMd5ΔMeq virus. The DEF-passaged rMd5ΔMeq construct at p19 was serially passage in DEF cell cultures. When plaque formation was advanced, cultures were trypsinized and transferred to new monolayers of DEF at 0.3 multiplicity of infection. Transfer intervals were about 7 days. Samples of infected cells were cryopreserved at every fifth passage starting at 20th until 50th (210 culture days), and these viruses were designated as Md5ΔMeq/p19, Md5ΔMeq/p30, Md5ΔMeq/p35, Md5ΔMeq/p40, and Md5ΔMeq/p50.

PCR amplification of MDV genes. To determine whether both copies of *meq* gene were absent in attenuated rMd5dMeq, we designed PCR primers to amplify *meq* and its flanking sequences. Primers used to amplify the *meq* gene were as follows: forward primer 5′-TGA ATG TCC CCT GTG AAG T-3′ and reverse primer 5′-CGG CAC TAT CGG TAC AAC A-3′. For detecting 132-bp repeat regions of MDV genome, the forward primer was 5′-GGA GAA AGT ATG TCG ATT TTA AAT GTA GTT-3′, and reverse primer was 5′-CTC GTA AGG CTT CCC GTC A-3′. DNA was extracted from attenuated rMd5ΔMeq-infected DEF using the Puregene DNA isolation kit (Qiagen, Valencia, CA). Procedures for PCR amplification of MDV *meq* gene or 132-bp repeat were as described previously (25,27).

Experimental design. *Commercial chickens.* The experimental design of the field test on commercial chickens depended on the availability of commercial chicks and facilities. The trial was carried out at a Hy-line International remote facility (Iowa) exclusively dedicated for MDV challenge studies. Groups of 200 commercial White Leghorn MAb + chickens of the Hy-Line W-36 variety were identified by wing bands and vaccinated at hatch by the subcutaneous route with doses of commercial vaccine preparations as per manufacturers' recommendations. The vaccine dose for different passages of rMd5ΔMeq was at 4000 plaque-forming units (PFU)/chick to be comparable to commercial dosage. Challenge was accomplished by placement of vaccinated chicks and nonvaccinated control chicks about 18 hr following vaccination in a brooder facility that contained 13,000–14,000 2- to 3-wk-old seeder chickens from a genetic susceptibility test that were inoculated with 500 PFU of 686 vv+ MDV at 1 wk of age. The nonvaccinated control chicks in the experiment were included to determine the protective index (PI) of vaccines. Morbidity and mortality from week 4 through 14 postvaccination was monitored and recorded. Chickens with typical symptoms and/or lesions were recorded as attributable to MD unless there was another obvious primary cause of mortality. The PI was calculated as the percentage of MD in unvaccinated, challenged controls less the percentage of MD in the vaccinated, challenged treatments divided by percentage of MD in unvaccinated, challenged controls × 100 (35).

ADOL chickens. The details for the pathogenesis and vaccine efficacy tests conducted in MAb− chickens were performed in ADOL SPF MAb-$15I_5 \times 7_1$ chickens as described previously (10). Briefly, at hatch, five groups of 17 chickens each were vaccinated with 2000 PFU of rMd5ΔMeq at p19, p35, p40, and p50, and CVI988/Rispens, with two additional groups of nonvaccinated chickens. At 5 days postvaccination, five groups of vaccinated and one group of nonvaccinated chickens were challenged with vv+ 686 MDV. The other group of nonvaccinated

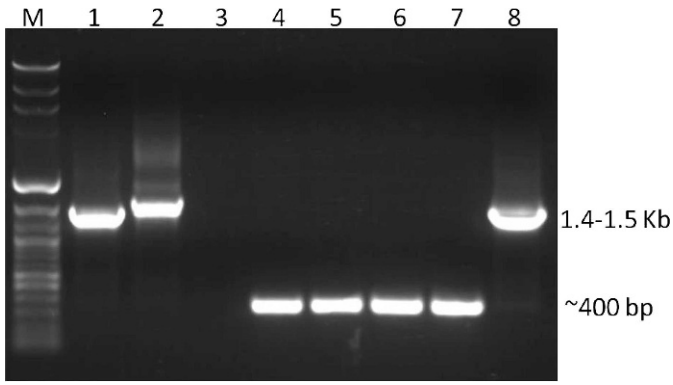

Fig. 1. PCR amplification of Meq gene. PCR products amplified from these extracted DNA samples: 1. BAC-rMd5; 2. CVI988/Rispens; 3. DEF; 4. rMd5ΔMeq/p19; 5. rMd5ΔMeq/p35; 6. rMd5ΔMeq/p40; 7. rMd5ΔMeq/p50; 8. rMd5. The wild type *meq* gene has 1.4–1.5 kb and *meq* null virus is 400 bp in size. M = molecular size in kilobases.

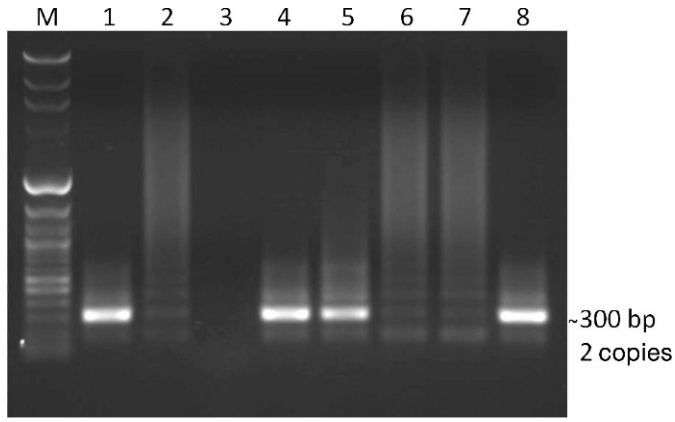

Fig. 2. PCR amplification of 132-bp repeat region of MDV genome. PCR products amplified from these extracted DNA samples: 1. BAC-rMd5; 2. CVI988/Rispens; 3. DEF; 4. rMd5ΔMeq/p19; 5. rMd5ΔMeq/p35; 6. rMd5ΔMeq/p40; 7. rMd5ΔMeq/p50; 8. rMd5. The 300-bp band indicates two copies of 132-bp repeats. The expanded repeats are not distinguishable and could indicate up to 20 or more copies of 132-bp repeats. M = molecular size in kilobases.

chickens served as un-inoculated control. Chickens that died during the trial or were euthanatized at the end of the experiment (10 wk) were necropsied and evaluated for gross lesions. To evaluate lymphoid organ weights, five chickens from each group were removed at 13 days postinfection, the lymphoid organs were collected, and relative organ to body weight was determined.

Statistical analysis. Duncan's multiple-range test was used to statistically determine the differences of body weight and bursa, thymus, and spleen over body weight ratios between the differently passaged virus groups. All analyses were performed using Statistical Analysis Software package version 9.2.1 (SAS Institute Inc., Cary, NC). Pairwise comparisons of the PI between vaccines was approximated using *z*-statistic for difference between proportion data (3) with Bonferroni corrections.

RESULTS

Absence of *meq* gene in attenuated virus. PCR was used to determine whether both copies of the *meq* gene were absent in rMd5ΔMeq virus after cell culture passage for 210 days. As shown in Fig. 1, BAC-rMd5, rMd5 and CVI988/Rispens had a band of 1.3 kb in size, whereas rMd5ΔMeq viruses from p19, p35, p40, and p50 had a 400-bp band indicating an absence of *meq* gene.

Detection of 132-bp repeat region of MDV. PCR was used to detect the presence of 132-bp repeat region in rMd5ΔMeq virus after cell culture passage. Both rMd5 and BAC-rMd5 were used since these two viruses had two copies of the 132-bp repeats (Fig. 2). Both the p19 and p35 of rMd5ΔMeq viruses showed two copies of 132-bp repeats similar to the parental virus rMd5. The 40 and 50 passage viruses showed multiple copies of 132-bp repeats similar to that of CVI988/Rispens virus, a typical pattern for attenuated viruses (26).

Cell culture passage of MDV eliminates lymphoid organ atrophy. This experiment was conducted in ADOL MAb− chickens. The rMd5ΔMeq/p19 virus was passaged from p19 to p50 in DEF culture in an attempt to alleviate its adverse effect on BTA (Fig. 3). The body weight of chickens inoculated with rMd5 and rMd5ΔMeq/p19 is significantly different from that of the control un-inoculated and high passage p35, p40, and p50 chickens. The ratio of bursa to body weight of rMd5, rMd5ΔMeq/p19, and p35 is significantly different from that of p40 and p50 ($P < 0.05$). Similarly, the ratio of thymus to body weight follows the same trend as that for bursa.

Protective efficacy of attenuated rMd5ΔMeq in commercial chickens. Groups of 200 each of W-36 Hy-Line chickens were used for this trial. The method of exposure for these vaccinated chickens to seeder birds was described in Materials and Methods. The seeder birds were inoculated with 500 PFU of vv+ 686 strain of MDV. The group of 200 unvaccinated chickens had 50% MD tumor incidence, whereas rMd5ΔMeq/p19, p36, p40, and p50 vaccinated birds had 3%, 18%, 21%, and 37%, respectively, corresponding to PIs of 94%, 64%, 58%, and 27%. On the other hand, CVI988/Rispens A had 20% and CVI988/Rispens B had 7% MD with PIs of 60% and 86%, respectively. The SB1 + HVT vaccinated chickens had 43% MD corresponding to PI of 14% (Fig. 4). This result is significantly different from that of the maternal antibody negative $15I_5 \times 7_1$ ADOL chickens (Fig. 5). As shown, the unvaccinated chickens challenged with vv+ 686 had 100% MD tumor incidence, whereas rMd5ΔMeq/p19, p35, p40, and p50 vaccinated birds had 15.4%, 13.3%, 20%, and 12.5%, respectively, corresponding to PIs of 80%–87.5%. On the other hand, CVI988/Rispens had 31.3% MD with a PI of 68.8%.

DISCUSSION

We have previously reported that rMd5ΔMeq/p19 is a superior vaccine to the commercial vaccines bivalent SB-1 + HVT and CVI988/Rispens. Recently, we also reported that the drawback of rMd5ΔMeq/p19 as a potential vaccine was the induction of BTA in MAb− chickens but not in MAb+ chickens (9). The parental rMd5 virus, however, induces BTA even in MAb+ chickens. Since rMd5ΔMeq/p19 is an effective vaccine, we attempted to improve the safety of this virus by serial passage in cell culture to eliminate its capacity to induce BTA. The present study was designed to investigate protective efficacies of cell culture–attenuated rMd5ΔMeq viruses in MAb+ commercial chickens and to determine whether BTA and protection efficacy are differentially regulated or tightly linked during attenuation.

In our earlier paper, chickens inoculated with p40 and p50 of the attenuated viruses showed no BTA, nor did those vaccinated and challenged with vv+ 686 virus (11). Thus, these attenuated *meq* null viruses also prevented the vv+ 686–induced BTA. Since the rMd5ΔMeq p40 virus did not induce BTA and could protect

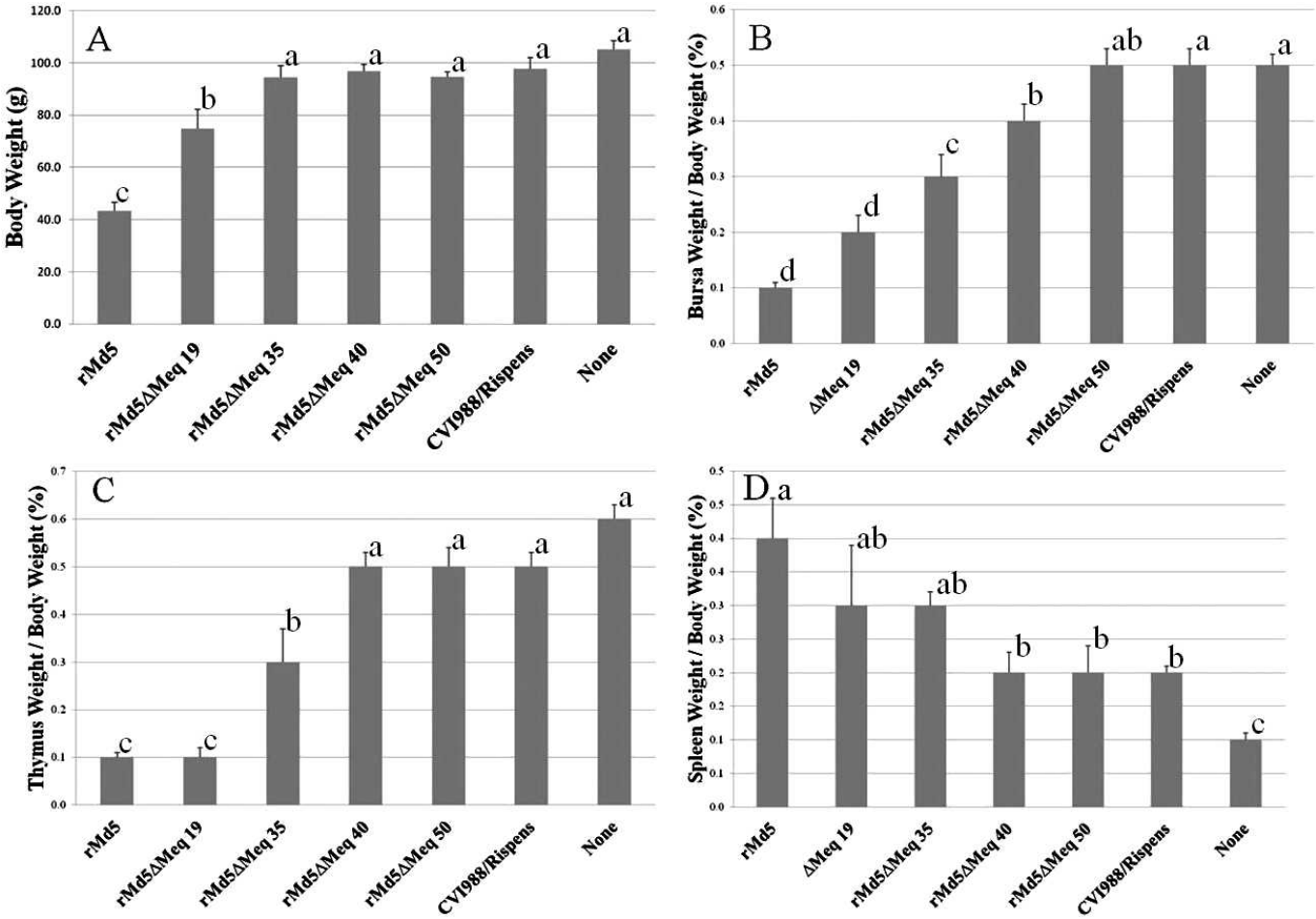

Fig. 3. Body and lymphoid organ weight of rMd5 and rMd5ΔMeq from p19 to p50. To evaluate lymphoid organ weights, five chickens from each group were removed at 13 days postinfection and the lymphoid organs were collected and relative organ to body weight were determined. (A) Body weight; (B) ratio of bursa to body weight; (C) ratio of thymus to body weight; (D) ratio of spleen weight to body weight.

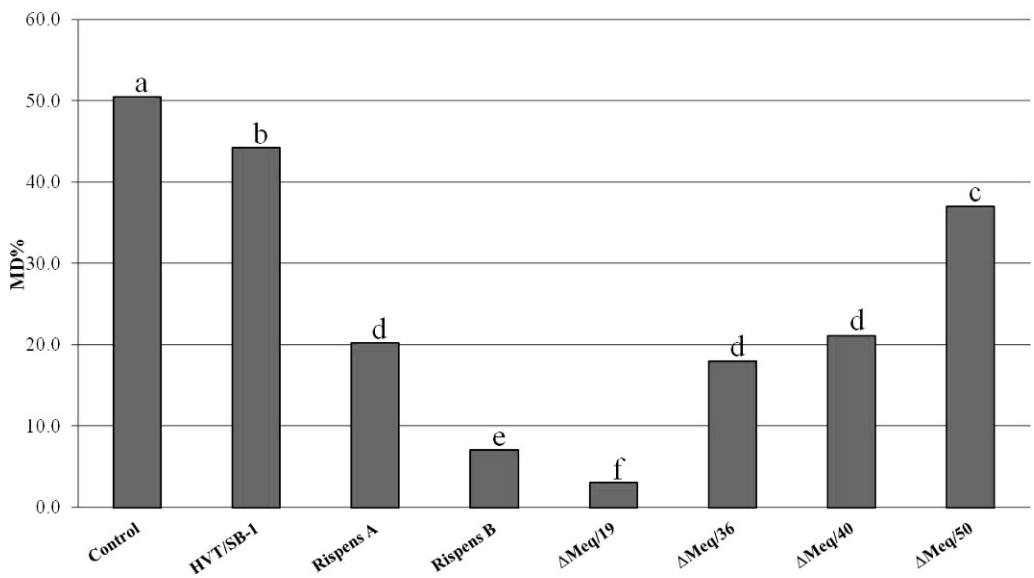

Fig. 4. Protective efficacy of attenated rMd5ΔMeq viruses in commercial chickens. 1. Unvaccinated control; 2. HVT/SB-1; 3. CVI988/Rispens A; 4. CVI988/Rispens B; 5. rMd5ΔMeq/p19; 6. rMd5ΔMeq/p36; 7. rMd5ΔMeq/p40; 8. rMd5ΔMeq/p50.

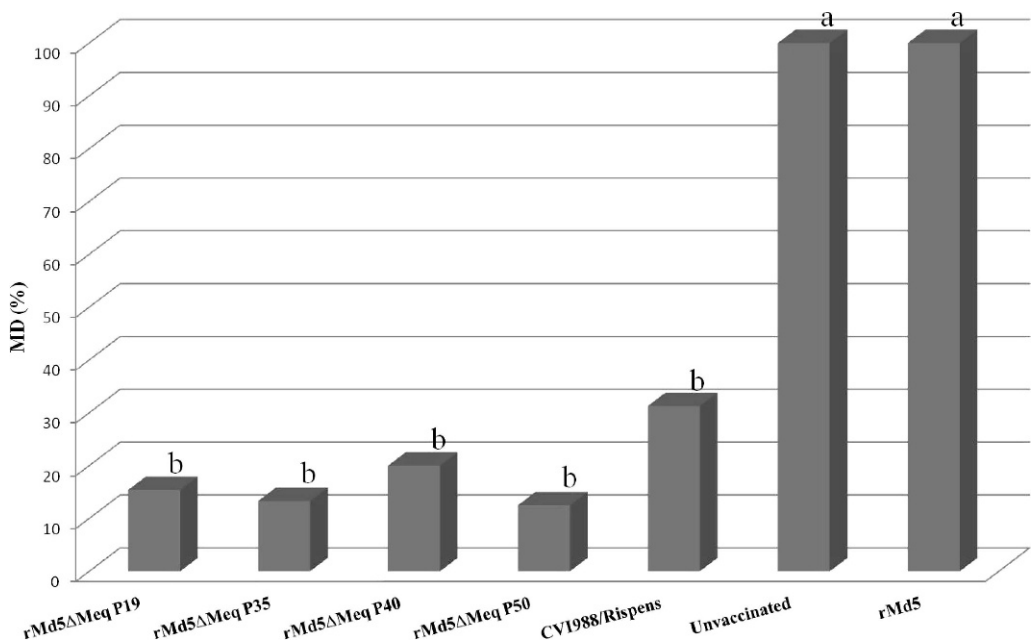

Fig. 5. Protective efficacy of attenated rMd5ΔMeq viruses in MAb− ADOL chickens. Five groups of 17 chickens each were vaccinated with 2000 PFU at 1 day of age and challenged at 5 days postvaccination. Chickens that died during the trial or were euthanatized at the end of the experiment (10 wk) were necropsied and evaluated for gross lesions. 1. rMd5ΔMeq/p19; 2. rMd5ΔMeq/p36; 3, rMd5ΔMeq/p40; 4. rMd5ΔMeq/p50; 5. CVI988/Rispens; 6. unvaccinated challenged with vv+ 686; 7. rMd5. This figure was based on data published by Lee *et. al.* (9).

MAb− chickens against vv+ 686 challenge, we decided to carry out a large-scale experiment with commercial chickens to see if the attenuated viruses could protect chickens under the field situation. The result showed that rMd5ΔMeq/p19 virus is a better vaccine than CVI988/Rispens, confirming our previous three trials in the Hy-Line chickens with a PI of 98–99 against challenge with vv+ 686 MDV. The PI of 94 observed in commercial chickens for rMd5ΔMeq/p19 is significantly better than CVI988/Rispens A (60%) or CVI988/Rispens B (84%). As the cell culture passage increased from 19 to 36, 40, and 50, the PI was reduced from 94% to 64%, 58%, and 24%, respectively. Therefore, the protective efficacies of these attenuated rMd5ΔMeq viruses in MAb+ commercial chickens are significantly lower than those from MAb− ADOL chickens (9). Several factors could account for the differences in protection from these two experiments: genetic lines of chickens (ADOL B-haplotype B^2B^{15} vs. W36 of mixed haplotypes); different challenge methods (day of age for Hyline vs. 5 days after vaccination for ADOL chickens); and MAb status of the chickens (MAb+ for Hyline and MAb− for ADOL). All these differences could contribute to the degree of protective efficacy observed. The effect of maternal antibodies on reduced vaccine efficacy has been well documented (6,24,34). MD vaccines of all serotypes provide lower levels of protection in chickens with maternal antibodies. In one study, protection by strain FC126 (HVT) was reduced by 39% in the presence of maternal antibodies (34). The maternal antibody to MDV could be the major factor in reducing vaccine efficacy in the commercial chickens.

The attenuation of virulent viruses by cell culture passage is a long and complex process with multiple mutations in the MDV genome (28). Many biological characteristics could alter during attenuation. Gimeno *et al.* (4) has considered biological characteristics that become attenuated at different times during serial passage as distinct and has proposed the term ''differential attenuation'' as a method for identifying characteristics that are not tightly linked. Examples are 1) the loss of A antigen (glycoprotein gC) is not associated with loss of virulence (15,16), 2) attenuation is not necessarily linked to poor *in vivo* replication (22,36), and 3) lymphoid leukosis-enhancing ability of a serotype 2 virus is attenuated with only minimal attenuation of protective efficacy (31). However, in our studies, this technique failed to discriminate between the properties of protection and BTA. Although both properties declined in parallel during serial passage and thus may be directly linked, it is also possible that the technique of differential attenuation was not sensitive enough to reveal differences.

The mechanism of attenuation is still not known. The cell culture passage that creates mutations in viral genome is well established (23,26,27,28). It was long believed that since MDV is serially passaged in cell culture, the virus becomes attenuated and the number of copies of the 132-bp repeat increases from 2 to often more than 20 copies, and this region was believed to be associated with virulence or attenuation (2,27). Recently, it was demonstrated that the rMd5 virus was still pathogenic after deletion of the 132-bp repeat sequences (26). Silva *et al.* (27) suggested that the expansion of the 132-bp region disrupts a 1.8-kb mRNA that has some similarity to the RNA for the fes/fps kinase related transforming protein; however, its role in MDV is not known. Recently it was shown that deletion of the 1.8-kb mRNA in MDV field strain GX0101 decreased the viral replication significantly but had no effect on its oncogenicity (29). Our PCR analysis of this repeat region in attenuated rMd5ΔMeq viruses showed two copies of 132-bp in p19, p30, and p35 and expanded copy numbers for p40 and p50. It is interesting that at p40, the expansion of the 132-bp coincided with its inability to infect lymphocyte and induced BTA (9). All these data taken together suggest that the 132-bp repeat is related to viral replication and BTA.

The mechanism of the linkage between attenuation, BTA, and protection may be related to viral replication rate *in vivo*. Our recent work demonstrated that chicken infected with rMd5ΔMeq/p19

virus induced abundant viral antigen pp38 in lymphoid organs and had a severe BTA. Chickens inoculated with attenuated p40 or p50 viruses had no detectable viral antigen, no detectable viral DNA, and no BTA (9). Therefore, BTA is the direct result of robust viral replication in lymphoid organs. It has been shown that for a vaccine to be effective, the virus must be able to replicate *in vivo*. In our study, the attenuated virus did not replicate *in vivo* and had reduced BTA and reduced protective efficacy. It is well known that cell culture passage has a deleterious effect on protection and our data confirm early reports on the effect of serial passage on protection (7,33,35,36,37). There is a narrow window in serial passage to produce good protection and beyond it could lead to over-attenuation and the virus becoming ineffective as a vaccine.

This study also showed that BTA and protection efficacy are tightly linked and both properties decline in parallel. It may or may not be possible to selectively attenuate the viral pathogenesis that induces BTA without an effect on its protection efficacy. Future sequence analysis of the genome of these attenuated viruses will hopefully elucidate the gene or genes involved in BTA or protection.

In conclusion, the attenuated rMd5ΔMeq/p40 has lost its capacity to cause BTA and is a good vaccine for MAb− ADOL chickens but less effective for MAb+ commercial chickens. However, this virus is as good as one of the currently used commercial vaccine (CVI988/Rispens), and it does not induce BTA. Our data further support the idea that BTA and protection are simultaneously attenuated by serial passage and these two properties are tightly linked.

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